Biochemical Pharmacology, Vol. 31, No. 17, pp. 2733–2738, 1982.
Printed in Great Britain.

0006-2952/82/172733-06 $03.00/0

RAPID OXIDATION OF NADH VIA THE RECONSTITUTED MALATE–ASPARTATE SHUTTLE IN SYSTEMS CONTAINING MITOCHONDRIAL AND SOLUBLE FRACTIONS OF RAT LIVER: IMPLICATIONS FOR ETHANOL METABOLISM

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(*Received* 2 *December* 1981; *accepted* 16 *March* 1982)

Abstract—In an attempt to assess whether hydrogen shuttle capacity might serve as the rate-limiting factor in the hepatic oxidation of ethanol, the malate–aspartate shuttle was reconstituted in systems containing mitochondrial and soluble fractions of rat liver. Oxidation of NADH was stimulated slightly by the addition of either glutamate or malate but when both substrates were added the stimulation was far stronger. This effect was greatly enhanced by aspartate indicating that, when not added to the system, extramitochondrial aspartate was limiting. It was found that the rate of oxidation of NADH was directly related to the amount of mitochondrial protein present but extramitochondrial reactions became restrictive when the 'soluble protein/mitochondrial protein' ratio fell below 0.8. When calculated on a whole tissue basis the maximum rate of oxidation of NADH by the reconstituted shuttle was substantially higher than reported rates of ethanol oxidation *in vivo*. The results are discussed in relation to the normal control of ethanol metabolism.

Our understanding of how ethanol metabolism is regulated in mammalian liver remains far from complete and although it is widely conceded that the overall rate of metabolism depends on the activity of alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1) there is disagreement on what factors are most important in determining the activity of this enzyme *in vivo* (for review see [1]). One theory [2–5] is that ethanol oxidation is limited by the rate at which cytosolic NADH is reoxidised by the various hydrogen shuttles that operate in the liver [6]. However, this theory is not universally supported and some workers believe that ethanol metabolism *in vivo* is governed not by the reoxidation of NADH but by the real level of alcohol dehydrogenase [7, 8].

One question in this debate which has not yet been satisfactorily answered is whether the hepatic hydrogen shuttles, and especially the malate–aspartate shuttle which is thought to be the most heavily involved in ethanol metabolism [9–11], have a maximum capacity exceeding that normally demanded by ethanol oxidation. If they do not then the argument in favour of their having a limiting role is strengthened. An adequate answer to this question can not easily be obtained from studies on intact cells but may be gained from studies on reconstituted systems. In experiments in which the malate–aspartate shuttle was reconstituted with rat liver mitochondria Cederbaum and co-workers [12, 13] and Lumeng *et al.* [14] found the maximum rate of oxidation of extramitochondrial NADH via the shuttle to be 10–30 nmoles/min per mg of mitochondrial protein at 30°. When these figures are converted to a whole liver basis and corrected to 37° they correspond roughly to the oxidation of 1–3 μmoles/min per g liver which is lower than the *in vivo* rate of ethanol metabolism (approx 4 μmoles/min per g liver [8, 10, 15, 16]). Even allowing for an appropriate contribution from other shuttles [9] the most obvious inference is that shuttle activity is likely to limit ethanol metabolism.

There are, however, technical reasons for questioning whether the reported values for malate–aspartate shuttle activity in reconstituted liver systems accurately indicate the true maximum capacity of the shuttle. In none of the studies were measurements made by a direct kinetic method and in one study [12] there was the added complication that the respiratory chain would probably have become limiting early during the incubation owing to the exhaustion of ADP. It was these shortcomings of the earlier work that prompted the current re-evaluation of the reconstituted malate–aspartate shuttle. To ensure precise determinations of the rate of NADH oxidation a direct spectrophotometric method was used and steps were taken to ensure that neither ADP nor oxygen was limiting. In an attempt to get closer to the physiological situation, a soluble fraction of rat liver was added to catalyse the extramitochondrial reactions of the shuttle. This is a departure from all past studies in which excesses of purified malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) and aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) have been added for this purpose. It was observed that, under these experimental conditions, added NADH was oxidised at rates at least double

those reported previously, throwing into question the suggestion that malate–aspartate shuttle capacity limits the rate at which the liver oxidises ethanol.

MATERIALS AND METHODS

Chemicals. Biochemical grade substrates and cofactors were obtained from Sigma Chemical Co. (St. Louis, MO), Boehringer-Mannheim Australia Pty. Ltd. (North Ryde, NSW, Australia) and CalBiochem-Behring Australia Pty. Ltd. (Carlingford, NSW, Australia). Other chemicals were AR grade.

Tissue preparations. Mitochondria were isolated from the livers of adult female Wistar rats by differential centrifugation essentially according to the procedure described by Bustamante *et al.* [17].

The soluble (or 'cytosol') fraction of rat liver was obtained as follows: diced liver was suspended in 1.5 vols. of ice-cold medium containing 250 mM mannitol, 50 mM triethanolamine buffer, pH 7.4, 1 mM ethylenediaminetetraacetate (EDTA), 2 mM $MgCl_2$ and 30 mM 2-mercaptoethanol (adapted from [18]) and was homogenised by two up-and-down strokes in a Potter–Elvehjem homogeniser. After centrifuging the homogenate at 30,000 *g* for 20 min the uppermost two-thirds of the supernatant was withdrawn and was centrifuged at 180,000 *g* for 1 hr. The clear supernatant fluid was carefully withdrawn by a Pasteur pipette, transferred to a dialysis sac and dialysed against 100 vols. of 50 mM triethanolamine buffer, pH 7.4, containing 1 mM EDTA, 2 mM $MgCl_2$ and 30 mM 2-mercaptoethanol. After 4 hr the dialysis medium was replaced with an equal volume of fresh medium and dialysis was continued for a further 15 hr. The dialysed liver extract was finally centrifuged at 20,000 *g* for 20 min to remove traces of particulate material and the resultant clear red supernatant, which contained approx 40 mg protein/ml, was retained as the soluble fraction. This preparation, unlike a normal post-mitochondrial supernatant, had very little innate NADH-oxidising capacity.

All preparative procedures were carried out at 0–4° and preparations were used in experiments the same day.

Reconstitution of the malate–aspartate shuttle. The buffered incubation medium contained 300 mM mannitol, 10 mM potassium phosphate buffer, pH 7.4, 10 mM Tris–HCl buffer, pH 7.4, 10 mM KCl and 5 mM $MgCl_2$ [12] with other additions as indicated. The medium was equilibrated with 100% medical-grade oxygen before use. All substrate solutions were adjusted to pH 7.4 before inclusion in the incubation medium.

To reconstruct the malate–aspartate shuttle the following standard procedure was adopted: into each of two spectrophotometer cuvettes was placed 2.29 ml of the oxygenated incubation medium containing 5 μmole of ADP and 0–10 μmole of L-aspartate. To each cuvette were added 0.05 ml of the mitochondrial suspension (1.4–2.2 mg of protein) and 0.1 ml of the soluble fraction (3.6–4.5 mg of protein). The cuvettes were placed in the thermostated (30°) sample and reference cuvette holders of a Varian DMS 90 UV-Vis recording spectrophotometer set to monitor absorbance at 340 nm versus time. After setting the baseline to zero and recording for approx 1 min, 0.01 ml of 38 mM NADH was added to the sample cuvette, causing the absorbance to rise to 0.96. The slow but steady fall in absorbance that followed was monitored for 2–3 min and then 0.05 ml of a solution containing 10 μmole of L-glutamate and 5 μmole of L-malate was added to both cuvettes. Absorbance readings were continued for periods up to 10 min following the addition of glutamate and malate, or until the absorbance had returned to near zero.

Variations to the standard procedure included the omitting of one or more of the substrates and the replacement of some or all of the mitochondrial suspension or soluble fraction with the respective media used in their preparation. All variations are described in the text but it is important to note that in each experiment the contents of sample and reference cuvette were identical except that the sample cuvette also contained NADH initially.

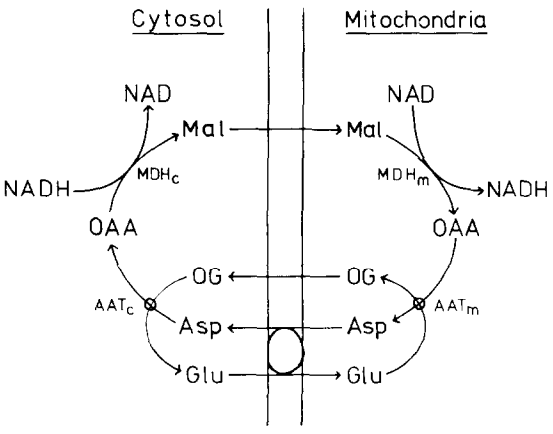
Respiration. The respiratory activity of each mitochondrial preparation was determined using a Rank Oxygen Electrode (Rank Bros., Bottisham, U.K.). The conditions were identical to those used in reconstituting the shuttle except that the medium was not pre-oxygenated and ADP was sometimes added after the substrates.

Protein estimations. Protein was measured by the method of Lowry *et al.* [19] with bovine serum albumin as the reference standard.

Statistics. Most results are expressed as the mean ± standard error of the mean for 3 or 4 separate experiments. Significance of difference was determined by the Student's *t*-test.

RESULTS

The malate–aspartate shuttle is illustrated in Scheme 1. To demonstrate unequivocally the operation of the shuttle it must be shown that the oxidation of extramitochondrial NADH is promoted by



Scheme 1. Transfer of reducing equivalents from cytosol to mitochondria via the malate–aspartate shuttle. Abbreviations: Mal, L-malate; OAA, oxaloacetate; OG, 2-oxoglutarate; Glu, L-glutamate; Asp, L-aspartate; MDH_c and MDH_m, cytosolic and mitochondrial malate dehydrogenases; AAT_c and AAT_m, cytosolic and mitochondrial aspartate aminotransferases.

Table 1. Stimulation of NADH oxidation by glutamate and malate in systems containing mitochondrial and soluble fractions of rat liver

Substrate added	Maximum rate of NADH oxidation (nmoles/min per mg mitochondrial protein) −Aspartate	+Aspartate
None	4.3 ± 0.8	5.1 ± 0.8
Malate	11.0 ± 0.6 (3)	13.5 ± 0.8
Glutamate	17.4 ± 0.3 (3)	26.4 ± 0.7
Glutamate + malate	47.1 ± 2.6	76.2 ± 2.7

Mitochondria (1.4–2.2 mg of protein) and soluble fraction (3.6–4.5 mg of protein) were incubated at 30° in 2.5 ml of oxygenated buffered medium containing 2 mM ADP and 0.15 mM NADH with or without L-aspartate (2 mM). The maximum rate of NADH oxidation was determined in the absence of added substrate and in the presence of 2 mM L-malate and/or 4 mM L-glutamate. Results are given as mean values ± S.E.M. for 4 separate experiments except that in two cases only 3 determinations were made as indicated in parentheses.

the two intramitochondrial substrates, glutamate and malate, in the absence of other shuttle intermediates. The results in Table 1 point to such an effect. In systems lacking aspartate, addition of malate caused only a very slight increase in the rate of NADH oxidation while addition of glutamate caused a larger increase, probably owing to the fact that it can serve as a source of malate via reactions of the tricarboxylic acid cycle. However, the stimulation brought about by glutamate and malate together was twice as great as the sum of their individual effects ($P < 0.01$). Table 1 also shows that although aspartate alone did not promote NADH oxidation, it significantly enhanced the response to glutamate with or without malate ($P < 0.01$ in each case) suggesting that, when not added, extramitochondrial aspartate was the limiting factor. It was also observed that, irrespective of the presence of mitochondria, the simultaneous presence of 2 mM aspartate and 2 mM oxoglutarate led to a very rapid rate of NADH oxidation (approx 125 nmoles/min per mg 'soluble' protein), though neither substrate alone had any effect.

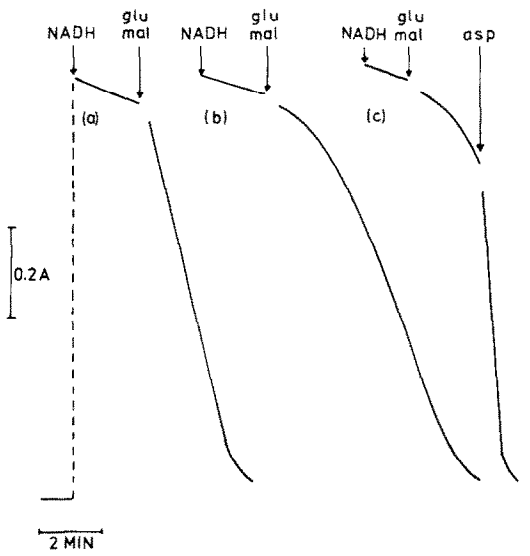


Fig. 1. Spectrophotometric recordings of the disappearance of NADH in systems containing mitochondrial and soluble fractions of rat liver together with components of the malate–aspartate shuttle. Reaction mixtures (final vol. 2.5 ml) consisting of oxygenated buffered medium containing mitochondria (1.4 mg protein), soluble fraction (3.9 mg protein) and 2 mM ADP were incubated at 30°. Additions made at the points indicated by arrows were 0.15 mM NADH, 4 mM L-glutamate (glu) and 2 mM L-malate (mal). Curve a, 2 mM L-aspartate present initially; curve b, no aspartate; curve c, 2 mM L-aspartate (asp) added after glutamate and malate as indicated. The traces depict the change in absorbance at 340 nm.

Typical curves depicting the time course of NADH oxidation under different conditions are shown in Fig. 1. The increase that followed the addition of glutamate and malate was most obvious when aspartate was also added (curve a), the maximum rate being attained very rapidly. If aspartate was not included (curve b) the maximum rate was not established until several minutes after the addition of glutamate and malate, indicating that the appearance of aspartate outside the mitochondria determined how quickly the increase in the NADH oxidation rate could be achieved. When aspartate was added about 2 min after the other substrate (curve c) there was an abrupt and very marked increase in the rate at which NADH was oxidised due to the rapid generation of oxaloacetate as the aspartate reacted with oxoglutarate already accumulated in the extramitochondrial space.

The results in Fig. 2 show the concentration-dependence of the aspartate effect. The broken line indicates the NADH oxidation rate 1 min after the addition of glutamate and malate whereas the continuous line shows the maximum rate achieved during the course of the incubation. It can be seen that the stimulatory effect of aspartate was much more marked at the 1 min interval but both curves show that the maximum effect was achieved with the concentration at 1 mM. It appears likely that once this situation is reached the supply of extramitochondrial oxoglutarate takes over as the limiting factor. Indeed, if 2 mM oxoglutarate was added to such systems there was an immediate and rapid increase in the NADH oxidation rate (data not shown).

In the results presented so far, the rate of oxidation of NADH is expressed on a 'per mg mitochondrial protein' basis, implying that mitochondrial reactions alone dictate the rate. However, it is conceivable that, in these systems, extramitochondrial reactions

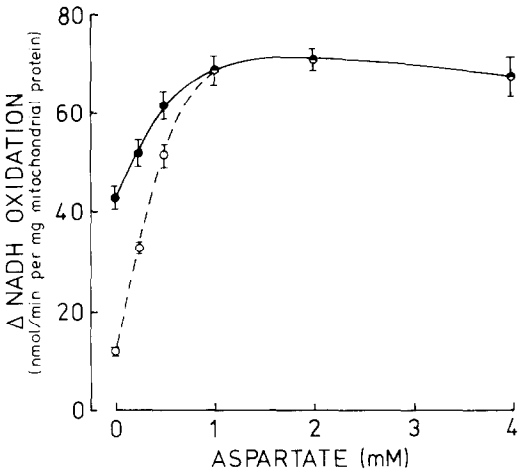


Fig. 2. Effect of aspartate concentration on the stimulation of NADH oxidation by glutamate and malate. Incubation conditions were as described for Table 1 with L-aspartate added at concentrations ranging from 0 to 4 mM. The rate of NADH oxidation was determined before addition of glutamate and malate, 1 min after addition of 4 mM L-glutamate and 2 mM L-malate, and after having reached its maximum. The curves show the increase recorded at the 1 min interval (○) and the maximum increase due to glutamate and malate (●). Points represent the mean values ± S.E.M. for 4 separate experiments.

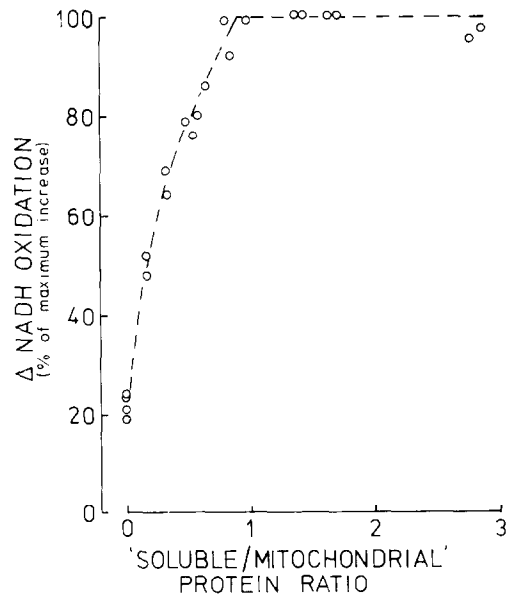


Fig. 3. Effect of varying soluble fraction on the oxidation of NADH via the malate–aspartate shuttle. Experimental conditions were essentially as described for Table 1 with 4 mM L-glutamate, 2 mM L-malate and 2 mM L-aspartate being added to all systems. The data are from 4 separate experiments in which the amount of mitochondrial protein in each system was between 1.4 mg and 2.2 mg but the amount of soluble fraction protein varied from 0 to 4.5 mg. Each point represents the glutamate plus malate-dependent increase in the NADH oxidation rate at the particular 'soluble/mitochondrial' protein ratio, expressed as a percentage of the maximum increase recorded in the same experiment.

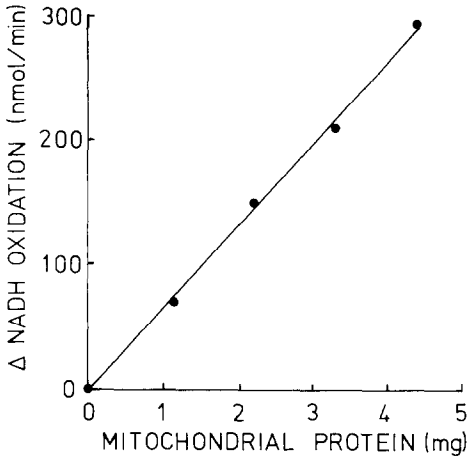


Fig. 4. Direct relationship between the rate of NADH oxidation and the amount of mitochondrial protein. Reaction mixtures (final vol. 2.5 ml) contained 2 mM ADP, 0.15 mM NADH, 2 mM L-aspartate, 3.6 mg of soluble fraction protein and from 0 to 4.4 mg of mitochondrial protein. Points represent the increase in the rate of NADH oxidation following the addition of 4 mM L-glutamate and 2 mM L-malate.

could be limiting, thereby making the units inappropriate. To determine which set of events limited NADH oxidation the amount of soluble fraction protein was varied while the amount of mitochondrial protein was held constant. The results in Fig. 3 show that when the 'soluble/mitochondrial' ratio exceeded 0.8 the rate was independent of the amount of soluble protein, but when the ratio fell below that value the extramitochondrial reactions became limiting. Residual shuttle activity was observed even in the absence of added soluble fraction showing that the enzymes responsible for the extramitochondrial reactions remained associated with the mitochondrial fraction, though at limiting levels. In the experiment presented in Fig. 4 the 'soluble/mitochondrial' ratio exceeded 0.8 throughout and a direct relationship was found to exist between the stimulation of NADH oxidation and the amount of mitochondrial protein. Hence the mode of expressing the NADH oxidation rate is legitimate provided appropriate experimental conditions are met.

An important facet of shuttle activity concerns its 'efficiency', which may be defined as the proportion of the mitochondrial respiration that is devoted to the oxidation of extramitochondrial reducing equivalents. Mitochondria incubated under the same conditions as those used in the reconstitution experiments exhibited a strong degree of respiratory control, the respiration rate rising from 19 ± 3 ng atoms oxygen/min per mg protein to 126 ± 6 ng atoms/min per mg protein (mean value ± S.E.M. for 4 experiments) when ADP was added. A similar dependence on ADP was demonstrated for the malate–aspartate shuttle; when reconstituted with aspartate but without ADP the increase in the NADH oxidation rate evoked by addition of glutamate and malate was only one-sixth of that recorded when ADP was present (data not shown). The 'efficiency' of the shuttle under state 3 respiratory conditions was calculated to be 56%, indicating

that most of the oxoglutarate formed in the mitochondria became available for extramitochondrial transamination.

DISCUSSION

The hypothesis that hydrogen shuttle activity plays a limiting role in the hepatic metabolism of ethanol has many adherents though the main evidence in its favour comes from studies with isolated hepatocytes from starved rats [3, 5, 20, 21] which have been shown to be deficient in shuttle intermediates [22, 23]. The hypothesis was also supported by the results of an earlier investigation into reconstituted hydrogen shuttles which suggested that the combined capacity of three major shuttles in the liver was just sufficient to mediate the oxidation of cytosolic NADH generated during ethanol metabolism [12]. However, careful analysis of that study reveals shortcomings that cast doubt on the validity of its estimates of the capacity of the malate–aspartate shuttle. A major problem was that, given the usual rate of respiration of liver mitochondria supplied with glutamate, malate and ADP ([24, 25] and current work), the ADP initially present would have been exhausted during the first half of the incubation period, severely curtailing the operation of the malate–aspartate shuttle during the second half. Also, the use of ethanol and alcohol dehydrogenase as a NADH-generating system, and the acceptance of ethanol disappearance as an estimate of shuttle capacity is dubious; experiments in this laboratory with similar systems show that the reaction catalysed by alcohol dehydrogenase quickly reaches equilibrium and the subsequent oxidation of ethanol depends on the removal of acetaldehyde as well as on the reoxidation of NADH (A. G. Dawson, unpublished observation). In this context it is interesting that Cederbaum and Rubin [26] found the rate of acetaldehyde oxidation by rat liver mitochondria to be almost identical to the rate of ethanol disappearance observed in their earlier study.

In the current work care was taken to avoid the problems noted above. The operation of the malate–aspartate shuttle in reconstituted systems was demonstrated by adding only glutamate and malate, thereby making the mitochondria responsible for providing the extramitochondrial oxidants as would occur *in vivo*. However, unless aspartate was also added it took several minutes for the maximum rate of NADH oxidation to become established. The elimination of this delay by aspartate indicated that a shortage of extramitochondrial aspartate was responsible. While several factors could contribute to such a shortage, the work of Papa *et al.* [24] and Murphy *et al.* [27] suggests that the intramitochondrial production of aspartate and its subsequent efflux should occur at rates sufficient to support the maximum rate of NADH oxidation observed in the current experiments. However, even if the formation of aspartate were stoichiometrically equal to the rate of oxygen consumption and all the formed aspartate were to enter the extramitochondrial space immediately, the consequent dilution is so marked in the reconstituted system that it would take about 10 min for the extramitochondrial aspartate to reach 1 mM, the concentration necessary for maximum shuttle activity. Hence it would be difficult, with this system, to demonstrate the maximum effect of glutamate and malate without also adding aspartate.

The inclusion in the reconstituted system of the soluble fraction of liver, rather than adding purified malate dehydrogenase and aspartate aminotransferase, permitted a limited analysis of the relative importance of the intramitochondrial and extramitochondrial reactions in determining shuttle activity. Because extramitochondrial reactions restricted NADH oxidation only when the 'soluble/mitochondrial' protein ratio fell below 0.8 it is unlikely that such reactions limit the capacity of the shuttle in intact liver tissue in which the ratio is about 1.6 [28, 29].

In this work the malate–aspartate shuttle was found to mediate the oxidation of up to 70 nmoles NADH/min per mg of mitochondrial protein, which is 2–7 times more than has been reported previously. Allowing for 60–65 mg of mitochondrial protein in each gram of liver [30, 31] the observed shuttle activity could account for the oxidation of 4.5 μmoles of extramitochondrial NADH/min per g liver at 30°. When adjusted to 37° this becomes 6–7 μmoles/min per g liver, almost twice the rate of ethanol metabolism *in vivo* [8, 10, 15, 16]. Hence this work gives no support to the notion that hydrogen shuttle capacity *per se* limits the rate at which ethanol is metabolised by the liver. On the other hand, it is recognised that the maximum activity of the malate–aspartate shuttle as determined in these systems might be far removed from its actual activity in intact tissue. Factors such as the levels of shuttle intermediates, the association of malate dehydrogenase with aspartate aminotransferase [32] and the channelling of oxaloacetate between them [33], and whether NADH produced by alcohol dehydrogenase has ready access to the enzymes of the malate–aspartate shuttle [34] could be of major importance in determining the true quantitative role of the shuttle in the hepatic oxidation of ethanol *in vivo*.

Acknowledgement—This work was supported by an Institute Research Grant from The New South Wales Institute of Technology.

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